***In vitro* cytotoxicity and *in vivo* sub-acute toxicity studies on *Vitex doniana* (Verbenaceae) leaves extract in MCF-7 breast cancer cells and Female Wistar rats**

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Abstract

Vitex doniana leaves are used in traditional medicine systems for the treatment of inflammatory related disorders and breast cancer in West Africa. However, there is a dearth of scientific evidence on the effectiveness of the plant leaves on cancer cells or on the safety of the plant if used continuously. This study examined the effects of *V. doniana* extract on the growth of MCF-7 breast cancer cells, B16-F10 melanoma cells and sEnd.2 endothelial cells. As well, the study investigated the effects of the extract on breast cancer cell morphology, cell cycle progression, migration and invasion, and levels of apoptosis regulatory proteins (Bad, PARP and p53). *In vivo*, the sub-acute toxicity of the extract in female Wistar rats was examined. The effects of *V. doniana* extract on MCF-7 cell growth, cell morphology and cell cycle were studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, light microscopy and flow cytometry respectively. The multi-target enzyme linked immunosorbent assay (ELISA) was used to study apoptosis proteins, and the XCELLigence system was used to measure cell invasion and migration. In the sub-acute toxicity study, female Wistar rats were administered 0, 50, 100 and 200 mg/kg of the extract daily for 28 days. Histopathology techniques were used to determine possible toxic effects of the extract on liver, kidney and mammary tissues. The MTT assay showed a concentration-dependent reduction in MCF-7 cell growth in the treated cells compared to the untreated control, and also inhibited the growth of melanoma cells. Furthermore, the results showed that the extract inhibited growth in cancer cells with more potency compared to the non-cancerous endothelial cells. Light microscopy revealed features of apoptosis in *V. doniana* -treated cells, while flow cytometry showed accumulation of MCF-7 cells in the G1 and sub-G1 phases of the cell cycle following treatment with the plant extract at 25 and 50 µg/ml respectively. *In vivo* sub-acute toxicity studies showed no significant ($p>0.05$) difference in serum alanine aminotransferase, gamma glutamyltransferase, urea and creatinine levels between the treatment groups and the control, while histopathological examination showed normal architecture of liver, kidney and mammary tissues across the groups. This study provides evidence that *V. doniana* has anti-cancer effects against MCF-7 breast cancer cells and exhibits no toxic effects in rats at the tested doses. Therefore *V. doniana* may have potential in the treatment of breast cancer, thereby necessitating further *in vivo* anti-cancer studies.

Key words: *Vitex doniana*; MCF-7; cytotoxicity; sub-acute toxicity; rats

Introduction

Breast cancer is responsible for morbidity and mortality among women, despite currently used therapeutic regimen (Balogun and Formenti 2015; DeSantis et al. 2015). Recent studies report relatively higher mortality rates in developing countries due to a number of factors among which are high treatment costs and inadequate cancer care facilities (Yip et al. 2015). Even for those who can afford orthodox cancer treatment, resistance by cancer cells to therapeutic agents and unwanted side effects observed among cancer patients are causes for concern. These challenges necessitate the development of therapeutic alternatives that are effective, locally available and with very little side effects (Ali et al. 2016).

Vitex doniana (Verbenaceae), commonly called black plum is the largest genius in the family *Verbenaceae*, which are deciduous shrubs distributed over Central Asia, the Mediterranean region and tropical Africa (Rani and Sharma 2013). *V. doniana* is used in herbal medicine for the treatment of a number of disorders including wounds, swelling and cancer (Agbafor and Nwachukwu 2011; Amegbor et al. 2012; Fadeyi, Fadeyi et al. 2013). Reported scientific investigations suggest that *V. doniana* has wound healing, anti-inflammatory and antioxidant properties (Amegbor et al. 2012; Adetoro et al. 2013). Oxidative stress and inflammation have been implicated in the promotion and progression stages of carcinogenesis and compounds with anti-inflammatory and antioxidant potency could have ameliorative effects in cancer (Reuter 2011). Plant preparations contain a plethora of compounds that could have anti-cancer, antioxidant and anti-inflammatory effects. However, one inadequacy of herbal preparations as used in traditional healing is certainty on the safety of the preparations especially during long term use (Makoshi et al. 2016). Some safety studies on the short-term use of *V. doniana* extract have been carried out, but there is inadequate information on the long term use of the extract in female Wistar rats. It is known that gender differences alter xenobiotic metabolism (Malhotra et al. 2009) resulting in varying toxicological outcomes.

Considering that there are no published works on the cytotoxicity of *V. doniana* in breast cancer cells despite the acclaimed therapeutic effects of the plant, and the dearth of information on the safety of long term use of *V. doniana* extract in female rats, this study examined the possible cytotoxic effects of the extract on MCF-7 breast cancer cells and the safety in female Wistar rats administered the extract for 28 days.

Experimental

Plant processing

The leaves of *V. doniana* were collected from a farm in Bauchi State, Nigeria and identified at the Federal College of Forestry, Jos with voucher number FHJ 189 obtained at the Herbarium for future references. The collected leaves were washed with distilled water and air dried at room temperature. The dried leaves were pulverized to obtain a dry powder which was subjected to ethanol extraction. Two hundred grams (200 g) of the obtained powder was transferred to labelled volumetric flasks and soaked in 1000 ml of 70 % ethanol for 48 hours. The mixture was

filtered using a 125 mm Whatman filter paper and the filtrate was allowed to freeze dry for 72 hours using a FD 8518 ilShin freeze dryer. The freeze-dried extract was then transferred into a labelled container for further analysis.

Cell culture

Estrogen receptor (ER)-positive MCF-7 breast cancer cells and melanoma B16-F10 cells were purchased from the American Type Culture Collection (ATCC, VA, USA). Endothelial sEnd.2 cells were obtained from Prof M.S. Pepper, Institute of Cellular and Molecular Medicine, University of Pretoria, South Africa. The MCF-7, sEnd.2 and B16-F10 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Whitehead Scientific, Cape Town, RSA) supplemented with 10% foetal bovine serum (FBS) (Sigma Aldrich, St Louis, MO, USA) and 1% penicillin-streptomycin (Sigma Aldrich, St Louis, MO, USA). Endothelial cells were further supplemented with 2mM glutamine (Mabeta and Pepper, 2009). All cells were incubated at 37°C in a humidified atmosphere containing 5 % CO_2. Cells growing in the exponential phase were used for testing.

Cell growth studies

The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**]** (MTT) assay was used to determine the effects of *V. doniana* on the growth of MCF-7 breast cancer cells. Exponentially growing MCF-7 cells were seeded into 96-well plates (5×10^3 cells/well) and allowed to attach for 24 h. The extract was prepared in 0.1 % dimethyl sulfoxide (DMSO). Cells were treated with 12.5, 25, 50 and 100 µg/ml of *V. doniana* extract and incubated for 48 h. These concentrations were chosen following screening of the extract and its fractions over 24-48 h. Cells in the control group were cultured in medium containing 0.1 % DMSO. After 48 h, the test compound/DMSO containing medium was removed from each well and the wells were washed with 100µl of PBS followed by the addition of 20 µl per well of the MTT reagent (5 mg/ml MTT in PBS) and the plate was incubated for 4 h at 37°C. The medium was removed and 100 µl DMSO was added and the absorbance measured using a micro plate reader at 540 nm (Mosmann 1983). Relative percentage cell viability = [(Absorbance of sample)/(Absorbance of control) × 100]. Each treatment was performed in triplicate. Colchicine, a drug that interferes with cell division via microtubule disassembly was used as a positive control.

Cell Morphology

The effects of *V. doniana* extract on MCF-7 morphology were examined using a previously described method (Mabeta et al. 2018) for hematoxylin and eosin staining. Exponentially growing MCF-7 cells were seeded into 6-well plates with heat-sterilised cover slips at the base (5×10^3 cells/well in 3 ml of medium) and allowed to attach for 24 h. The plant extract was prepared in DMSO and serially diluted with medium to obtain appropriate concentrations. After 24 h, the medium was removed from each well and the cells were treated with 0, 25 and 50 µg/ml of *V. doniana* extract and incubated for 48 h. Medium was removed, and Bouin's fixative was added for 30 min. The fixative was discarded and 70% ethanol was added for 20 min, after which the cover slips were rinsed in tap water. Mayer's Haemalum was added for 20 min and rinsed in tap water for 2 min, and the cells were rinsed with 70 % ethanol. A solution containing 1 % eosin was then added to the cells for 5 min, after which the cells were

rinsed twice for 5 min with 90%, 96%, 100 % ethanol and then with xylol. The cover slips were mounted on microscope slides with resin and allowed to dry after which they were examined using a microscope (Nikon E100 LED binocular microscope, Nikon, Tokyo, Japan). Images were obtained using a camera (Nikon D3400 DSLR camera).

Cell cycle Analysis by FACS (Fluorescence-Activated Cell Sorting) Analysis

MCF-7 cells were seeded at density of 1 × 10^6 cells/well in 6-well plates. After 24 h, cells were treated with *V. doniana* at concentrations of 25 (IC_{50} concentration) and 50 µg/ml (double the IC_{50} concentration). After 48 h of treatment, cells were washed with PBS and harvested using trypsin, and fixed with ice-cold 100% ethanol overnight. The fixed cells were centrifuged for 1 h at 300 × g and washed with PBS twice. The cells were then treated with 50 µl of 100 µg/ml RNase A solution in PBS and incubated at 37ºC for 15 min. Thereafter, 400 µl of 50 µg/ml propidium iodide (PI) solution in PBS was added and then vortexed (Mabeta et al. 2018). The cells were incubated for at least 10 minutes to stain in the dark before being analysed using a Beckman FC 500 Series Flow Cytometer (Beckman Coulter Life Sciences, CA, USA). Propidium iodide fluorescence was detected on the FL3 channel (excitation/emission 496/633 nm). A minimum of 10,000 events for each sample were acquired.

Multi-target apoptosis ELISA assay

Apoptosis proteins (Bad, caspase 3, PARP and p53) were measured using the PathScan Apoptosis Multi-Target Sandwich ELISA Kit (Cell Signaling Technology, USA) according to the manufacturer's instructions. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and treated with fraction at 25 µg/ml or 0.1 % DMSO for 24 h. The cells were lysed with lysis buffer, and the protein concentrations were measured to confirm equal loading onto an ELISA plate. The absorbance was read at 450 nm using the BioTek ELx800 Plate Reader (BioTek® Instruments, CA, USA).

Cell invasion and migration

Cell invasion experiments were performed using 96-well Boyden chamber with an 8 µm polyethylene terephthalate (PET) membrane coated with matrigel. MCF-7 cells (1×10^5) were seeded into the upper chamber containing medium with 30 or 300 µM DFO, 300 µM DFO + 100 µM DPI, or 300 µM DFO + 100 µM PD98059. After 24 h of incubation, the cells were fixed with 0.2% glutaraldehyde and stained with 0.1% crystal violet. The number of cells that migrated to the underside of the membrane was counted under a light microscope. A 1% solution of triton X-100 was added to each well and the absorbance was read at 570 nm using a microplate reader (BioTek® Instruments, CA, USA). Experiments were performed three times.

Sub-acute Toxicity Study of *V. doniana* extract in female Wistar rats

Ethics

Ethical approval was sought and obtained from the Ahmadu Bello University Ethical Committee (Reference number: ABUCAUC/2018/004) in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Experimental Animals

A total of twenty (20) healthy female Wistar rats aged 180 ± 10 days weighing 165 ± 10 grams were purchased from the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The animals were acclimatized for a period of 1 week under ambient environmental conditions in well aerated cages at the Department of Anatomy Animal House, Ahmadu Bello University, Zaria. They were allowed free access to grower's mash (Vital feeds, Grand Cereals Plc) and water ad libitum.

Study Design

Twenty (20) rats were randomly divided into four groups of five (5) rats each. Group 1 served as control and received distilled water while Groups 2, 3 and 4 received 50, 100 and 200 mg/kg of *V. doniana* extract respectively. The treatment was administered for 28 days.

Preparation of serum and tissue samples

Twenty four (24) hours after the last administration, the rats were sacrificed humanely and blood was collected in non-heparinized tubes and allowed to stand after which centrifugation was done at 10,000 × g for 10 min to obtain serum, which was stored in labelled containers. Liver, kidney and mammary tissues were excised and stored in buffered formalin at 25°C.

Serum markers of Tissue Damage

Serum alanine aminotransferase (ALT) was determined according to the method of Reitman and Frankel, 1957 by monitoring the concentration of pyruvate hydrazine formed with 2,4-dinitrophenylhydrazine. The reaction mixture contained 0.1 ml of diluted sample, phosphate buffer (100 mmol/l, pH 7.4), L-arginine (100 mmol/l) and α-oxoglutarate (2 mmol/l). The mixture was incubated for 30 min at 37°C. Next, 0.5 ml of 2,4-dinitrophenylhydrazine (2 mmol/l) was added to the reaction mixture and allowed to stand for 20 min at 25°C. Subsequently, 5.0 ml of NaOH (0.4 mol/l) was added to stop the reaction and absorbance was read against the reagent blank after 5 min at 546 nm. The corresponding concentration was obtained from a standard curve for ALT.

Serum gamma glutamyltransferase (GGT) was determined according to the method of Szasz (1969). A volume of 0.1 ml of diluted sample was mixed with 0.5 ml of tris buffer (100 mmol/l, pH 8.25) and incubated at 25°C. The mixture was carefully shaken and 0.5 ml of L-γ-glutamyl-3carboxy-4-nitroanilide (2.9 mmol/l) was added. The

reaction mixture was mixed, initial absorbance read, and then absorbance values were recorded after 1, 2 and 3 min at 405 nm wavelength. The following equation was used to determine the corresponding GGT concentration

GGT (U/l) = 1158 X dA 405 nm/min

Urea in serum was determined according to the method of Searcy et al. 1967. Three cuvettes for blank, standard and sample were used. For the blank, 10 µl of distilled water was added to 100 µl of picric acid (35 mmol/l). The cuvettes for standard contained 10 µl of standard and 100 µl of picric acid (35 mmol/l), while the cuvette for sample contained 10 µl of sample and 100 µl of picric acid (35 mmol/l). The three cuvettes were then mixed and incubated at 37ºC for 10 min. After that, 2.5 ml of Sodium hydroxide (0.32 mol/l) and Sodium hypochlorite (27 mmol/l) were added to the three cuvettes, mixed and incubated at 37ºC for 15 min. Absorbance of sample and standard were then read against the blank at 546 nm. Urea concentration was calculated using the formula below:
Urea concentration (mmol/l) = (Absorbance of sample)/(Absorbance of standard) X Standard concentration
Serum creatinine was determined according to the method of Moss et al. 1975. 1 ml of picric acid (35 mmol/l) and Sodium hydroxide (0.32 mol/l) was pipetted into a cuvette and 0.1 ml of sample was added, mixed and absorbance read after 2 and 4 min. The same was done for standard but without addition of sample. All absorbance was read at 492 nm. Creatinine concentration in mg/dl was calculated as change in absorbance of sample/change in absorbance of standard × standard concentration (mg/dl)

Histology

Histological examination on tissues was carried out by hematoxylin and eosin staining (H&E) according to the method of Fischer et al. 2008. Sections of liver, kidney and mammary tissue fixed in 10% buffered formalin were dehydrated in 95% ethanol, followed by 100% ethanol, cleared in xylene and then embedded in paraffin. Micro sections (3 µm) were then prepared, stained with hematoxylin and eosin and examined under a microscope (Nikon E100 LED binocular microscope). Images were obtained using a camera (Nikon D3400 DSLR camera).

Statistical analysis

The quantitative results are expressed as mean ± standard deviation unless stated otherwise. At least three replicates were analysed for each treatment dose. One-way analysis of variance (ANOVA) and post-hoc Tukeys test were used to determine the differences among the means. Values of $P < 0.05$ were considered to be statistically significant.

Result and Discussion

Growth inhibitory effects of *V. doniana* on MCF-7 cells, B16-F10 melanoma cells and sEnd.2 endothelial cells

The *V. doniana* extract was tested on three cell lines (MCF-7, B16-F10 and sEnd.2). The extract reduced MCF-7 cell growth in a concentration-dependent and time-dependent manner, with an IC_{50} value of 24. 91 ± 0.2 µg/ml in MCF-7 breast cancer cells. However, the IC_{50} value in sEnd.2 normal endothelial cells was 48. 93 ± 1.6 µg/ml. Thus, the effects of the extract were more pronounced in cancer cells than in endothelial cells (Table 1). The effect

of *V. doniana* treatment on MCF-7 cell growth was significantly different ($P<0.05$) from the microtubule destabilizer colchicine at 50 µg/ml. Since the plant has been used in West Africa to treat breast cancer, this study focused on further investigation of the plant extract in the MCF-7 breast cancer cell line. Indeed, the pronounced reduction in the percentage survival of MCF-7 cells following treatment with *V. doniana* (Figure 1) suggests the plant extract had cytotoxic effects. These observations are similar to previous findings on the cytotoxicity of plants (Kho et al. 2015).

Cell morphology

The control cells (C) showed normal morphology, and some cells were dividing (Figure 2). Cells treated with 25 µg/ml *V. doniana* (A) showed cells in interphase, while cells treated with 50 µg/ml *V. doniana* (B) had a round morphology, some showed cell shrinkage and nuclear condensation which suggests that the extract may induce apoptosis at these concentrations. A round morphology, altered membrane integrity and DNA condensation are associated with apoptosis (Ghobrial et al. 2005). To investigate these observations, cell cycle analysis was undertaken.

Cell Cycle Analysis

Cell cycle analysis after 48 h showed that compared to control, a significant number ($P < 0.05$) of MCF-7 cells treated with 50 µg/ml *V. doniana* were in the G_0 phase, while a significant number ($P < 0.05$) of MCF-7 cells treated with 25 µg /ml of the extract were in G_1 phase (Figure 3). Cell cycle arrest at G_0/G_1 phase is associated with apoptosis, and accumulation of cells at G_0/G_1 phase delays the entry of cells into mitosis which is responsible for cell division (Pucci et al. 2000; Aliyu et al. 2013). The inhibition of MCF-7 cell growth by *V. doniana* at 50 µg /ml might involve the induction of apoptosis (Figure 3). Therefore, apoptosis was investigated employing the multi-target apoptosis ELISA kit.

Apoptosis

To further investigate apoptosis induction in MCF-7 cells treated with *V. doniana*, proteins that play a role in apoptosis induction, Bad, caspase 3, PARP and p53, were measured using a multi-target apoptosis detection kit. Results showed an increase in p-53 phosphorylation as well as increased PARP-cleavage following the treatment of MCF-7 cells with the extract (Figure 4). When cleaved, PARP is an executer of apoptosis. In addition, the extract increased the levels of phosphorylated Bad, a member of the Bcl2 family (Figure 4). When phosphorylated, Bad forms heterodimers with anti-apoptotic proteins Bcl-xL and Bcl-2, thus preventing their apoptosis suppressive role (Aranovich et al. 2012). Caspse-3 activity was not detected, possibly due to the fact that MCF-7 cells do not express caspase-3 (Janicke, 2009). Therefore, the extract may have promoted apoptosis through PARP cleavage and Bad phosphorylation.

Effect of *V. doniana* extract on MCF-7 cell invasion and migration

Treatment of MCF-7 cells with the extract showed that *V. doniana* inhibits MCF-7 cell invasion and migration, although the extract was more potent at higher concentrations of 50 µg/ml (Figure 5). The migration of cancer cells,

and their invasion of the extracellular matrix are important for metastasis (Mierke, 2011). Thus the extract could have a role in decreasing the occurrence of metastasis, however, further studies need to be undertaken using an animal model of breast cancer.

Effect of sub-acute administration of *V. doniana* on some serum markers of liver damage

There was no significant difference in serum alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) activity between the treatment groups (Table 2). This suggests that administration of *V. doniana* extract did not have deleterious effects on the liver at the doses of this study. Serum ALT and GGT are used as markers of liver damage, because damage to hepatocytes during exposure to certain concentrations of xenobiotic can cause increased serum levels of ALT and GGT (McGill 2016).

Effect of sub-acute administration of *V. doniana* on some serum markers of kidney damage

There was no significant difference in serum urea and creatinine levels between the treatment groups (Table 2), suggesting that the treatment did not cause damage to the kidney of the rats at the doses of this study. Serum urea and creatinine are used to estimate glomerular filtration efficiency (Ferguson and Waikar 2012). Any condition that causes damage to the nephrons affects kidney reabsorption, resulting in decreased clearance of urea and creatinine (Wasung et al. 2015).

Effect of sub-acute administration of *V. doniana* on Liver Histology

Histological examination of the liver of rats administered *V. doniana* extract showed normal hepatic sinusoids, quite similar to control (Figure 6). Hepatic sinusoids are important blood vessels of the liver which could become dilated or congested during xenobiotic induced toxicity (Furlan et al. 2016). The absence of such alterations in the examined liver tissues suggests that *V. doniana* administration for 28 days did not cause damage to the liver at the doses of this study, and is consistent with the insignificant difference in ALT and GGT levels between the groups (Brancatelli et al. 2018).

Effect of Sub-acute administration of *V. doniana* on Kidney Histology

Histological examination of kidney of the rats administered *V. doniana* extract did not show any tubuloglomerular lesions (Figure 7). Kidney damage is associated with glomerular atrophy and distension of the renal glomerulus capsular space in rats (Ding et al. 2011). The normal architecture observed in the groups is consistent with the insignificant difference in urea and creatinine levels between the groups, suggesting safety of the extract to kidney of the rats at the doses of this study.

Effect of Sub-acute administration of *V. doniana* on mammary tissue histology

Histological examination of the mammary tissue in the *V. doniana* administered rats did not show histopathological alterations (Figure 8). The mammary gland is composed of a glandular ductal network, lined by a protective layer of

epithelial cells which show hyperplasia during damage that causes inflammation (Wen and Aguirre-ghiso 2014). The normal architecture of mammary tissue seen from the histology results suggest that administration of *V. doniana* did not cause damage to normal breast tissue at the doses of this study.

Conclusion

Findings from this study suggest that *V. doniana* is cytotoxic to breast cancer and melanoma cells as it had growth inhibitory effects on these cells. Given the prevalent use of *V. doniana* for the management of breast cancer in West Africa, further mechanistic studies were undertaken using breast cancer cells. At IC_{50} concentrations of 25 µg/ml, the plant extract arrested MCF-7 cell division, and most of the cells were in interphase, while at double the IC_{50} concentration the extract arrested cells in the sub-G_1 phase, a phenomenon associated with apoptosis. Further studies confirmed that the extract induces apoptosis in MCF-7 cells by enhancing p53 activity. Furthermore, *V. doniana* extract decreased the migration and invasion of breast cancer cells, even at low concentrations, processes associated with the metastatic dissemination of cancer cells. Important to note is that there was a comparatively lower growth inhibitory effect observed in non-cancerous endothelial cells exposed to the *V. doniana* extract, suggesting that the extract is more cytotoxic to cancer cells than non-cancerous cells. In healthy rats, the sub-acute toxicity study showed that administration of *V. doniana* extract for 28 days did not have any deleterious effects on liver, kidney and mammary tissues of the experimental rats. Thus, *V. doniana* may have potential in anti-cancer treatment given its *in vitro* growth inhibitory effects on breast cancer cells and its apparent lack of toxicity *in vivo*. However, further research on the *in vivo* anti-cancer effects of *V. doniana* is required to establish therapeutic efficacy and optimal doses for ER-positive breast cancer.

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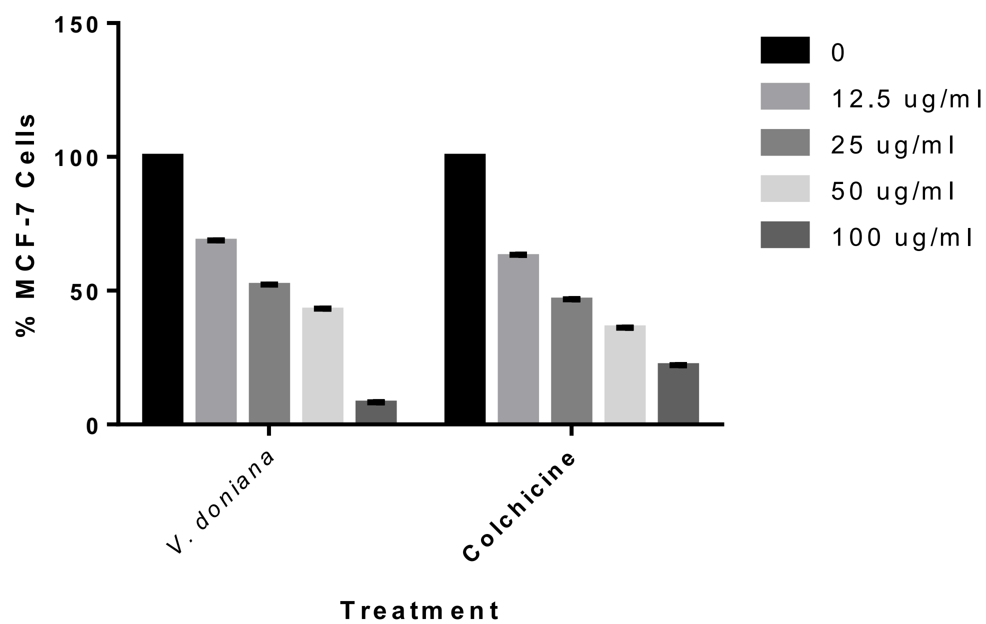
FIGURES



Results are presented as mean values ± SD of five replications

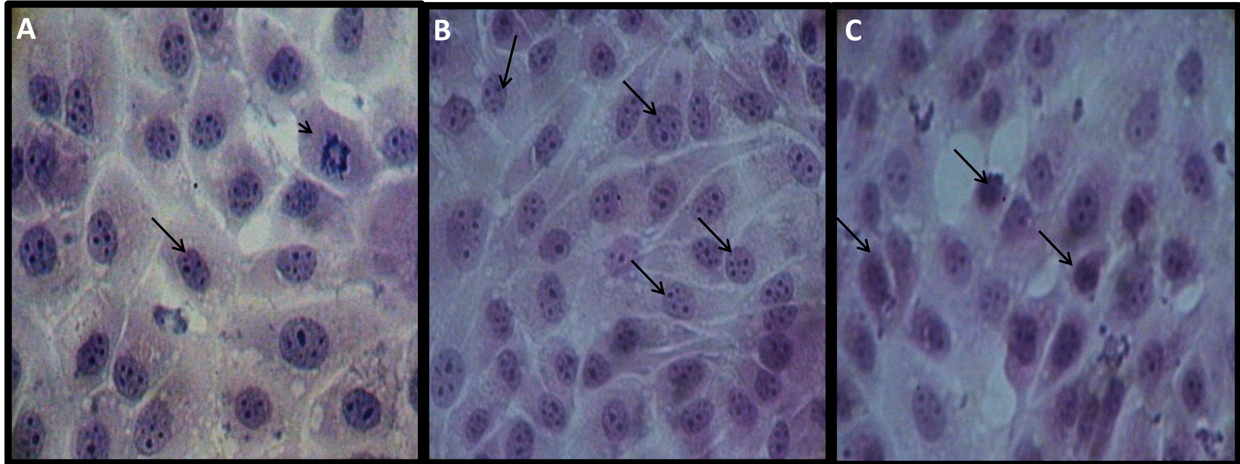


Figure 2: Effect of *V. doniana* on MCF-7 Morphology

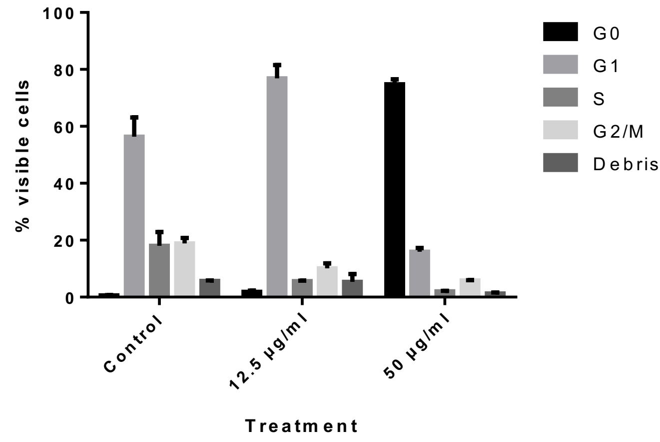
Magnification - × 400

A – Control, the short arrow indicates a cell in metaphase, the long arrow showing cells in interphase

B- Cells treated with *V. doniana* (25 µg/ml), the long arrows indicate cells in interphase

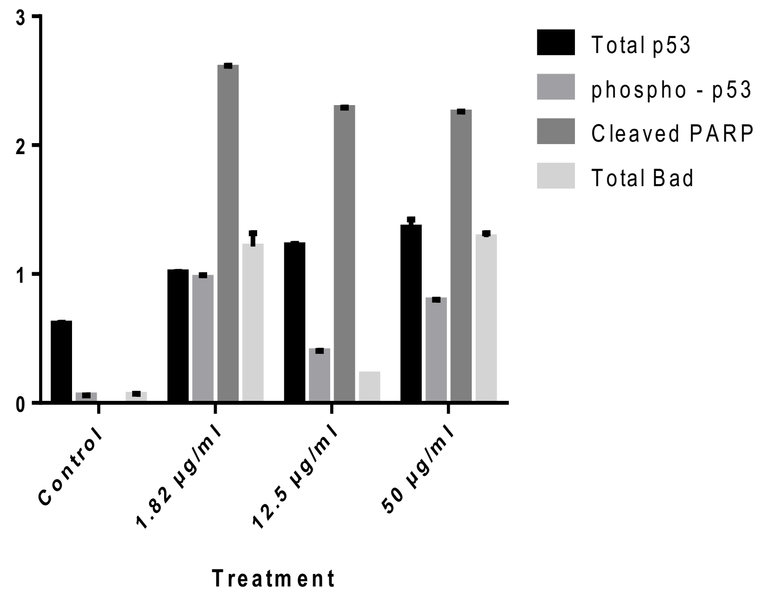
C - Cells treated with V. doniana (50 µg/ml), long arrow indicates hypercondensed chromatin

Figure 3: Distribution of MCF-7 cells in different Cell Cycle phases after treatment with *V. doniana*

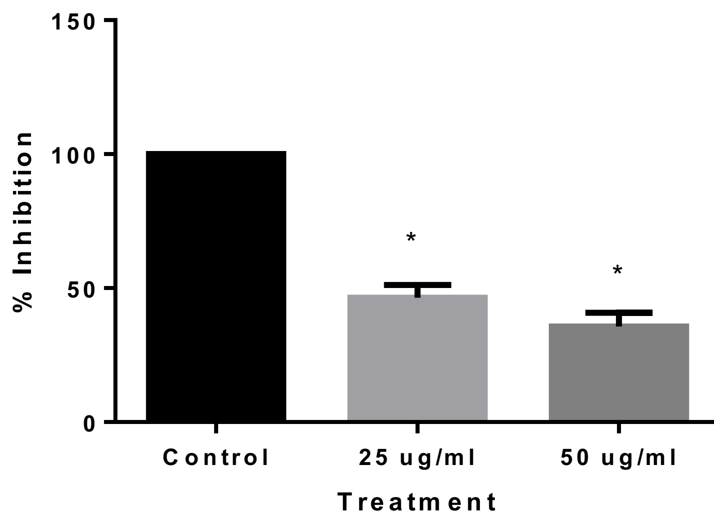


Results are presented as mean values ± SD of five replications

Figure 4: Levels of apoptosis regulating proteins after treatment of MCF-7 cells with V. doniana.



Results are presented as mean values ± SD of three replications



Results are presented as mean values ± SD of three replications

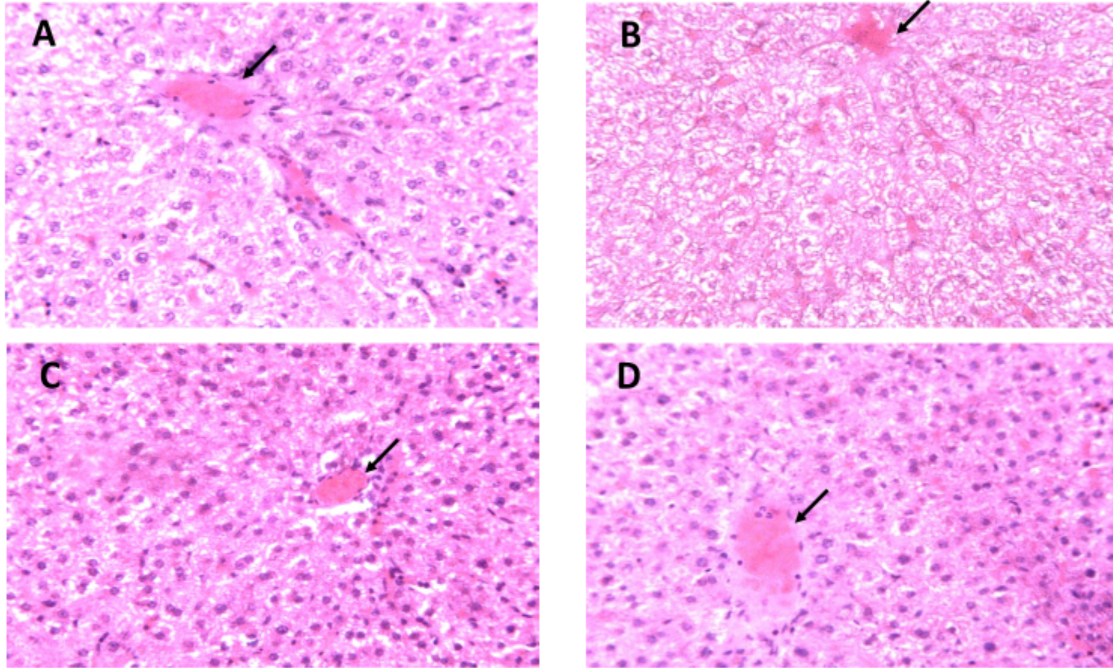


Figure 6: Effect of Sub-acute administration of *V. doniana* on Liver Histology

Magnification - × 400

A - Liver histology of control

B - Liver histology of 50 mg/kg *V. doniana* administered (sub-acute) group

C -Liver histology of 100 mg/kg *V. doniana* administered (sub-acute) group

D - Liver histology of 200 mg/kg *V. doniana* administered (sub-acute) group

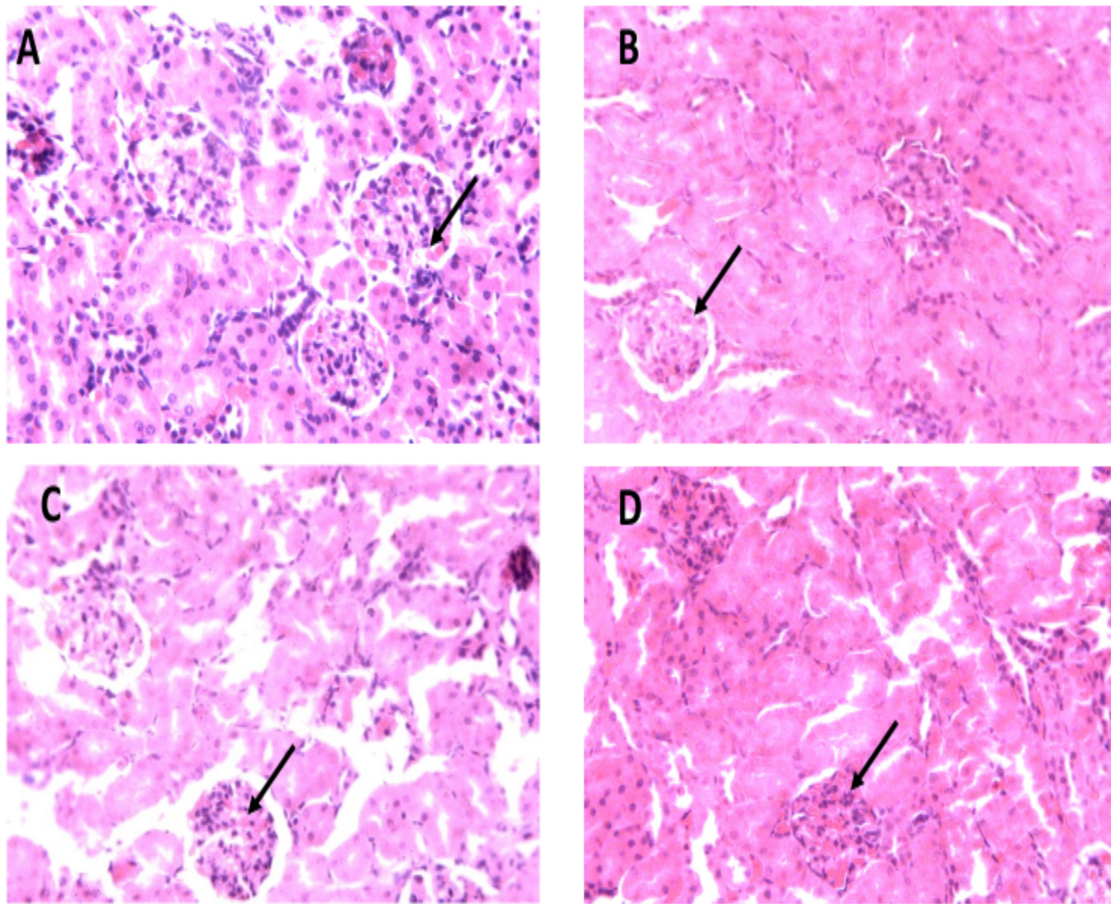


Figure 7: Effect of Sub-acute administration of *V. doniana* on Kidney Histology

Magnification - × 400

A - Kidney histology of control

B - kidney histology of 50 mg/kg *V. doniana* administered (sub-acute) group

C - kidney histology of 100 mg/kg *V. doniana* administered (sub-acute) group

D - kidney histology of 200 mg/kg *V. doniana* administered (sub-acute) group

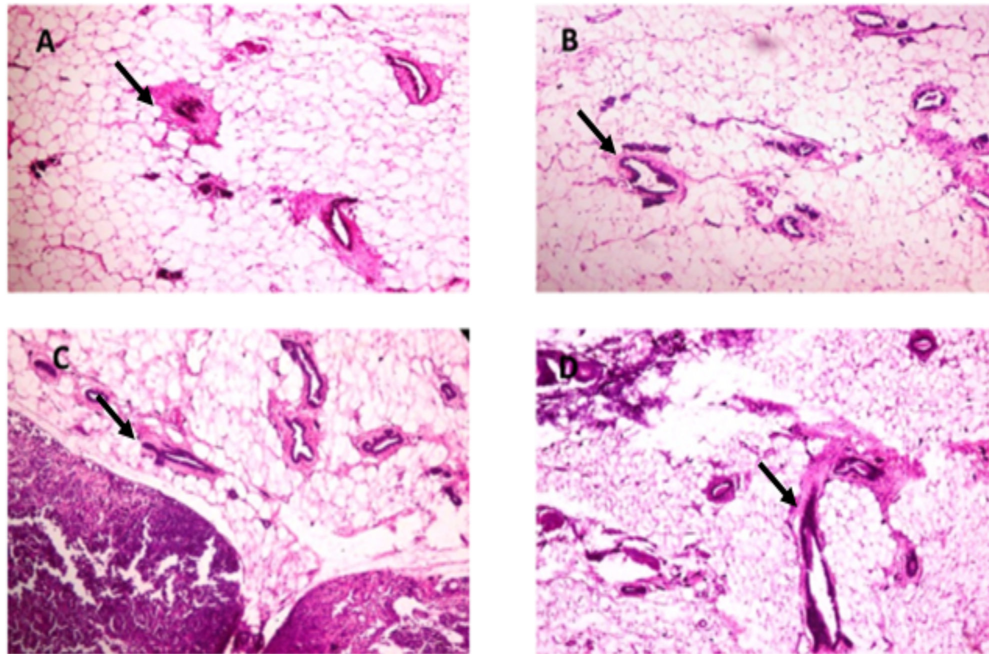


Figure 8: Effect of Sub-acute administration of *V. doniana* on mammary tissue histology

Magnification - × 100

A - Mammary tissue histology of control

B - Mammary tissue histology of 50 mg/kg *V. doniana* administered (sub-acute) group

C - Mammary tissue histology of 100 mg/kg *V. doniana* administered (sub-acute) group

D - Mammary tissue histology of 200 mg/kg *V. doniana* administered (sub-acute) group

TABLES

Table 1: IC_{50} Values of Cells Treated with *V. doniana*

Cell line	**IC_{50} value (μg/mL)**
MCF-7	24. 91 ± 0.2
B16-F10	19.65 ± 2.1
sEnd.2	48. 93 ± 1.6

Values are presented as mean ± SD of five replications

Table 2: Effect of sub-acute administration of *V. doniana* on serum markers of tissue damage

	Alanine aminotransferase (IU/L)	Gamma glutamyltransferase (IU/L)	Urea (mmol/L)	Creatinine (µmol/L)
Control	49.2 ± 3.03[a]	1.67 ± 0.14[a]	6.81 ± 0.52[a]	64. 69 ± 6.83[a]
50 mg/kg *V. doniana*	48.2 ± 5.81[a]	1.59 ± 0.12[a]	6.67 ± 0.83[a]	64. 85 ± 2.53[a]
100 mg/kg *V. doniana*	48.4 ± 2.88[a]	1.57 ± 0.12[a]	6.57 ± 0.42[a]	65.26 ± 7.32[a]
200 mg/kg *V. doniana*	47.4 ± 2.61[a]	1.56 ± 0.12[a]	6.35 ± 0.37[a]	69.11 ± 3.54[a]

Values are mean ±SD of five replications

[a] represents $P>0.05$